Structural advances on titin: towards an atomic understanding of multi-domain functions in myofilament mechanics and scaffolding

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Running Title: Structure of titin

Abstract

Titin is a gigantic filamentous protein of the muscle sarcomere that plays roles in myofibril mechanics and homeostasis. 3D-structures of multi-domain fragments of titin are now available that start revealing the molecular mechanisms governing its mechanical and scaffolding functions. This knowledge is now being translated into the fabrication of self-assembling biopolymers. Here we review the structural advances on titin, the novel concepts derived from these and the emerging translational avenues.

KEYWORDS

Titin / multi-Ig tandem / protein scaffold / crystal structure / protein nanofibre

Introduction

Titin is an intra-sarcomeric protein of striated muscle that plays complex roles in myofibril mechanics and mechanosensing, homeostasis and development (reviewed in 1-2). The single titin molecule (>3MDa) spans >1 μm *in situ*, from the sarcomeric Z-disc to the central M-line (3). Despite its diverse and intricate functionalities, titin has a simple modular architecture, consisting primarily of Ig and FnIII domains (~300 domains) linked in tandem and amounting to over 90% of its mass. Domain tandems are interrupted only occasionally by unique sequences that include the elastic PEVK-rich region and the pseudokinase TK domain (4) (**Fig 1**). Titin integrates mechanical and signalling roles in the sarcomere. In its mechanical function, it generates passive tension through the straightening of its I-band springs during myofibrillar stretch. As a scaffold, it supports a rich interactome (Tcap, CARP/MARP, MuRF1, myosin, MyPB-C, myosin, obscurin, obscurin-like 1 and myomesin, among many others), being a central node in myofibrillar signalling. Thus, titin is regarded as a biomechanical platform that integrates the proteostatic response of the myofibril to mechanical and metabolic stress (1-2).

Despite significant advances in understanding the cellular functions of titin, elucidation of the filament structure has been slow (structural data recently reviewed in 5). In recent years, the first multi-domain structures of titin fragments have become available that shed light on its functional mechanisms. Structures comprise representatives from the primary functional regions of titin: Z-disc, I-band, A-band and M-line. Several structures of titin components complexed to cellular partners are now also available. Together, these structures glimpse into the principles of domain arraying in the chain and illustrate its molecular strategies to specifically recruit binding partners to defined loci. Finally, the growing knowledge of titin is now leading to the nanofabrication of bioscaffolds of great promise. Here, we review the knowledge on titin as an integrated protein chain where functionalities emerge from the joint action of its components.

Historical view: Titin as the sum of its individual domain components

The early view of titin considered its domains as 'beads on a string'. This perspective was founded on early electron microscopy (EM) images that showed no apparent order in the molecule (6-7). The known role of the I-band in elasticity led to an immediate focus on the architecture and dynamics of its Ig-tandems (**Fig 1**). Biophysical characterisation of constructs containing two to six domains revealed weak domain interactions through small interfaces, supporting the 'beads on a string' viewpoint. Prior to establishing the key role of the PEVK region in elasticity, Ig stretch-unfolding (later termed the *secondary elasticity of titin*; 8) was thought to drive the mechanical response of titin. However, this is now a controversial concept and Ig stretch-unfolding is no longer regarded as a primary mechanism of titin elasticity *in vivo* (9-10). Nevertheless, the potential of stretch-unfolding to serve as a rapid mode of regulation through the S-glutathionylation of cryptic cysteines in Ig still suggests a physiological significance (11).

Multi-domain structures reveal a dynamic higher-order in the titin chain

The foundations for an improved understanding of titin structure-function have been laid recently with the availability of the first multi-domain structures of titin

segments. Given the colossal size of titin, its atomic structure can only be studied as short fragments. But even those have proven a challenge for NMR and X-ray crystallography methods, which are troubled by their intrinsic flexibility. Nonetheless, several multi-domain 3D-structures are now available, namely: Z1Z2 (Z-disk) (12); I65-I70 (I-band) (13); A77-A78 (14), A164-A165 (A-band) (PDB: 3LCY); and A168-A170 (M-line) (15) (an extended list of fragments, sub-fragments and mutated variants is given in **Table S1**).

Multi-domain structures reveal three primary types of domain interfaces (**Fig 2**): *i) loose* connections, where domains are joined by hydrophilic three-residue linkers that, being free from interactions, impose low steric restrictions on dynamics and allow large modular motions (representative pairs are Z1Z2, I65-I66 and I66-I67); *ii) restrained* connections, where domains are joined through ultra-short linkers (0-2 residues) and sustain a small number of direct domain-domain contacts. This results in sterically restricted domain motions (as in I67-I68, I68-I69, I69-I70, A168-A169); and *iii) tight* connections, where domains are joined through zero-length linkers and hold multiple interdomain contacts that result in an apparently rigid junction (as in A77-A78).

All interface types appear to favour extended domain arrangements (**Fig 1)**. But domain pairs linked through short connections - *restrained* or *tight* – also show a strikingly regular up-and-down domain geometry roughly corresponding to a modular torsion angle of ~180^{o} (a range of 156^{o}-176^{o} observed across pairs). This appears to be the most sterically favourable way to tightly pack Ig/FnIII folds sequentially in a chain. This packing only permits narrow cones of motion between neighbouring domains and causes a local reduction in chain dynamics – predictably, the formation of collapsed V-conformations (where modules close onto each other) is energetically disfavoured in this case. It could be concluded that *restrained* and *tight* interfaces define sterically favourable domain arrangements and have certain tautness. In contrast, *loosely* connected domains can undergo modular motions of large amplitude and adopt collapsed V-arrangements at low energetic cost. Yet, a V-closure has only been observed in the crystal structure of Z1Z2, where it was stabilized by a cross-linking metal ion and co-existed with a conventional extended form (**Fig 1**). An exhaustive study that used SAXS, NMR and MDS suggested that the closed conformation in Z1Z2 is rare and rapidly exchanges with the extended state (8, 12). The predominance of these extended arrangements likely stems from the fact that the integrating domains do not interact laterally with each other, so that V-conformations are not sustained in the absence of a stabilizing stimulus - e.g. compression force or the binding of a ligand. Thus, titin can be regarded as a modular system, sterically managed and locally adaptable.

Crystal structures thus show that domain interfaces in titin are heterogeneous in their mechanical plasticity. Interestingly, interface types are not randomly distributed along the chain. In titin, Ig and FnIII modules are largely organized into super-repeats (particularly in the myosin-binding A-band and the differentially-spliced I-band spring, **Fig 1**) and so are their interfaces as indicated by sequence conservation patterns (14,16 and references within). This has led to propose that titin behaves as a segmented chain, where segments (of variable length) with reduced conformational freedom are interspersed with flexor points (13). This chain model has been supported by EM images of a recombinant 19-Ig segment (I39-I57) of the differentially-spliced I-band (13), which show rod-like segments interrupted by sharp kinks. It might be speculated that *tautness* and *segmentation* is the cause of the coiling and dash-like features also observed in extracted, full-length titin molecules (e.g. 7).

Torsional energy in the titin chain: tertiary elasticity and molecular shape memory

Titin's elasticity resulting from the straightening of its I-band springs is termed *tertiary elasticity* (8). The Ig-tandem region lengthens during small stretch, but this does not result in measurable passive tension. It is the unraveling of the PEVK spring at moderate to high stretch that causes an increase in tension (9,10). Z1Z2 is the only Ig pair experimentally observed in a range of conformations (**Fig 1**), thereby allowing an exploration of conversion paths between extended and collapsed states. Studies (12) have shown that domain closure in Z1Z2 follows a helical motion and is achieved by a simple torsion around a single main chain bond, in this case in the alanine residue of the AET linker sequence. To gain a further insight into the energy landscapes of mechanically induced conformational transitions in Ig-tandems, the structures of Z1Z2 and I65-I70 were subjected to steered MDS (8,17,18). These structures contain pairs separated by *loose* (Z1Z2, I65-I66, I66-I67) and *restrained* connections (I67-I68, I68-I69, I69-I70). It was found that all linkers allowed a variability of conformations between neighbouring domains, but that twisting motions (particularly in *restricted* interfaces) were limited, with a notable energy barrier at ~70^{o}-90^{o} (suggesting a high resistance to domain "flipping" in the chain). It was also observed that at high stretch, the tension developed in the fully straightened chain was the result of bond rupture at domain interfaces and modular torsions, suggesting a certain enthalpic contribution to elasticity. This has led to propose a modified, entropic-enthalpic worm-like chain model of titin elasticity that better fits experimental stretch profiles (19). Myofibrils shorten during force generation, but also twist and rotate and undergo diverse deformations. It remains to be investigated in which physiological scenarios titin might store energy as a restrained torsional spring.

Protein binding on specific titin loci: Sterically modulated scaffolding

Revealing the set of interactions held by titin in the sarcomere and their dependence on local conformations of the chain is essential to understand the integration of stretch-response and signalling. In this regard, structures of titin components complexed with cellular partners are now available: Z1Z2 bound to Tcap/telethonin (20), the Z-repeat 7 (Zr7) in complex with the C-terminal EF-hands (EF34) of α-actinin (21), and the M-line Ig domain M10 bound to Ig1 from obscurin (Obs) (22) and obscurin-like protein 1 (ObsL1) (23-24) (**Fig 1; Table S1**).

The Z1Z2:Tcap and the M10:Obs/M10:ObsL1 complexes at the N- and C-terminus of titin, respectively, play a structural role in sarcomere assembly. These structures have been the focus of a recent commentary (25) and will not be discussed here in detail. Briefly, the complexes exploit a binding mechanism known as β-sheet augmentation, where an intermolecular β-sheet is formed across subunits. In Z1Z2:Tcap, a Tcap molecule sandwiches itself between two antiparallel Z1Z2 segments acting as a biological "glue" that fuses two titin molecules. Tcap is unstructured in isolation but folds into an antiparallel β-sheet upon binding to Z1Z2. In the three complexes, subunit fusion relies largely on main chain interactions. This binding mode is sturdy and offers enhanced resistance to point mutations that could otherwise disrupt the ultrastructure of the sarcoskeleton and lead to disease.

Titin is also capable of engaging helical proteins, but the only such complex characterized structurally to date is ZR7:α-actinin^{EF34} (21). The linkage of titin and α-actinin in the Z-disc contributes to the organization of actin filaments and to transmit tension across sarcomeres during muscle function. The interaction involves the 45-residue Z-repeat motifs of titin and the 73 C-terminal amino acids of α-actinin, which contain the non-calcium binding EF-hand 3 and 4 (EF34). Free Zr7 in solution is unstructured. Upon binding to EF34, it acquires an α-helical conformation that mediates binding via a cluster of small hydrophobic residues. A recent study of full-length α-actinin shows that the Zr7:EF34 binding induces and selectively recruits the open conformation of α-actinin (26).

Although structurally uncharacterised, the complexation between A168-A170 (N-terminal to TK) and the E3 ubiquitin ligase MuRF1, linked to myofibril turnover, has been demonstrated *in vitro* (15,27). The structure of A168-A170 has an extended, stiff architecture, characterized by a shallow surface groove that spans its full length and is predicted to dock the C-terminal coiled-coil-like domain of MuRF1. The groove has a unique 9-residue loop protrusion in its mid point, corresponding to an insertion in A169 between β-strands A and A'. Mutagenesis showed that this loop is deterministic in MuRF1 binding. A169 is thus an example of domain individualization in titin. It was known that MuRF1 binding requires at least two domains: A168-A169 (27), but recent data revealed that the true binding site comprises also TK and confirm that no single domain can bind MuRF1 (4). The Ig-Ig-Fn-TK scaffold (estimated length 18-22 nm) fits the rod dimensions of MuRF1 revealed by EM (~17±3 nm) (28). Interestingly, our recent work on the close TK homolog twitchin kinase from *C. elegans* (29) suggests that the Fn-TK junction comprises a mechanically deformable structural element. It is therefore reasonable to expect a coupling of MuRF1 binding to this region and mechanosensing.

Taken together, the multi-domain binding sites in Z1Z2:Tcap and A168-TK:MuRF1 indicate that long-range domain conformations sterically contribute to partner recognition. An alteration of the twist and hinge opening of domains in the chain by stretch, post-translational modification or binding of accessory proteins could modulate binding affinities, enabling or hindering binding. Further, the malleability of folding/unfolding transitions appears to be an important element in the energetics of titin scaffolding. Both Tcap and Zr7 are unstructured in isolation and fold upon binding of their partners. An additional strategy for securing specific binding is the speciation of individual domains, as observed in A169. Interestingly, the Ig domain from Obs involved in the M10:Obs complex also has individual features, but these are not exploited in the binding. Further structural studies are required to reveal the general significance of these and other binding strategies in titin.

Titin as a source of novel nanomaterials

Progress in the molecular understanding of titin allows translating its unique properties into biotechnological applications. Titin is a rich source of intracellular Ig fold variants that, unlike Ig components of antibodies, are remarkably stable and undemanding to produce recombinantly in bacteria. Studies of Z1 have shown it to tolerate the drastic reengineering of its CD loop, permitting grafting into the fold recognition motifs of 9-14 residues length that remain accessible and bioreactive (30). Thus, titin domains can act as effective scaffolds for peptide display.

Further research has been directed at the organization of titin's Ig into nano-objects with higher order. In this respect, the tandem I65-I70 has been displayed as a compact monolayer on liposomes using metallochelation (31). The organization of Ig into nanofibres has also been achieved. For this, the associative properties of Z1Z2 and Tcap have been exploited in the engineering of chimeras that self-assemble spontaneously in solution to yield nanofibers (~13 nm in diameter) (32) (**Fig 3**). In this polymer, assembly interfaces and functionalization sites are spatially segregated permitting the "bottom-up" incorporation of bioactive polypeptides without affecting fibre morphology. Functionalization of the fibre with attachment sites for Ni^{2+}-NTA containing gold-nanoparticles has confirmed nanopatterning at □5 nm intervals that yielded a regular linear array over micrometres (32). This polymer has high potential for stoichiometrically controlled orthogonal functionalization, presenting 6 independent modification sites in its building block. It holds great promises to selectively bind and organize matter in the fine nanoscale. Finally, an 8-mer of domain I91 has been recently used in the generation of a hydrogel with potential to sense force-triggered redox activity (33). It can be expected that as the atomic knowledge of titin evolves, its functions in muscle will be further translated into smart biomaterials with controlled functions.

Conclusion

Structural data suggest that the functional roles of titin are assisted by a subtle inter-domain order, derived from short linkers that sterically govern domain packing and dynamics in the chain. Steric regulation is also a likely factor modulating the affinity of titin-associated proteins for composite receptor sites in the chain. Thus, modularity constitutes a simple but effective structural means to integrate the mechanical and scaffolding functions of titin in the myofibril.

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FIGURE LEGENDS

Figure 1: Structural studies of titin at a glance
Schematic representation of titin (repeats at the I- and A-band are indicated) accompanied by available 3D structures (*upper*) and a list of characterized fragments (*below*; their characterization method is indicated. Only recombinant fragments of known composition are listed). The several conformations of Z1Z2 observed to date (ranging from the extended to the collapsed state) are shown. In complexed structures (*right, upper*), the titin component is in blue and the partner protein in red. As M10:Obs and M10:ObsL1 are closely related, only one structure is shown in representation of both complexes. It is noticeable that I-band and M-line regions have been foci of attention.

Figure 2: Domain interfaces in the titin chain
Representatives of **a.** *loose*; **b.** *restrained*; **c.** *tight* interfaces. Directed contacts within a domain adopt the colour of the domain; contacts across domains or involving linker residues are in red.

Figure 3: Titin-based biopolymer
a. Z1Z2Z1Z2 chimera that assembles by Tcap complementation in a self-propagating mode. EM images of the resulting fibres in **b.** a native form in isolation; **c.** in a fibre population**; d.** and **e.** bound to gold-nanoparticles (from 32).

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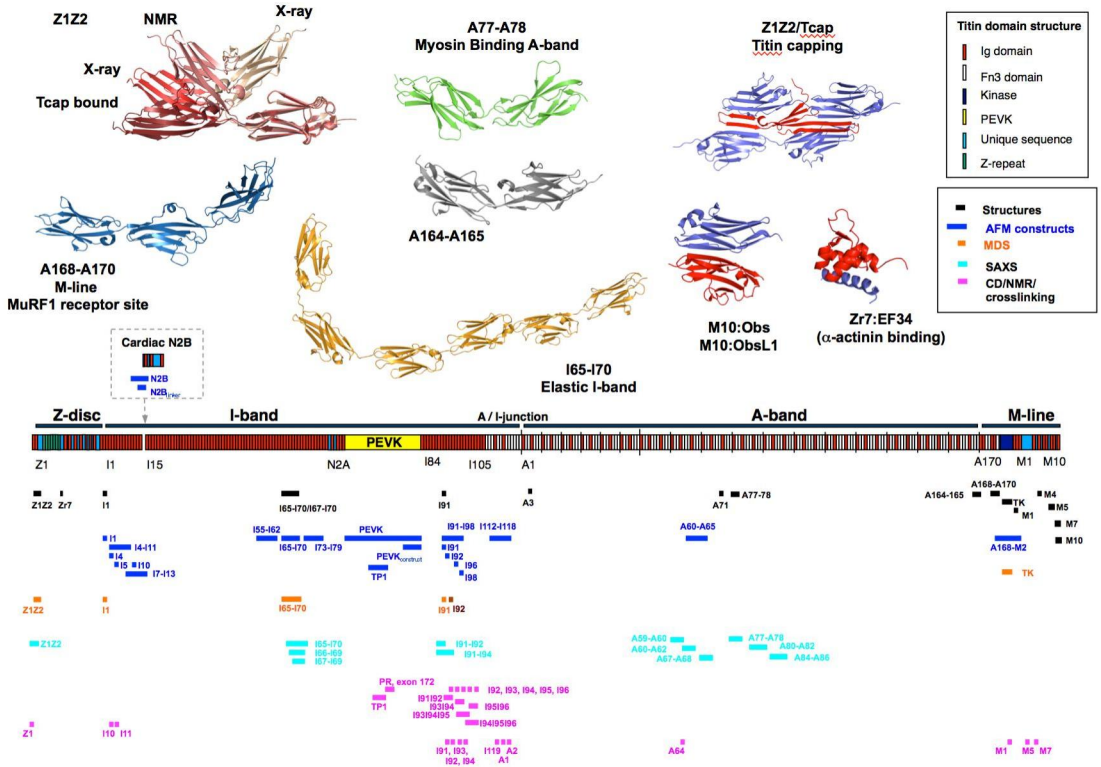
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Elastic I-band
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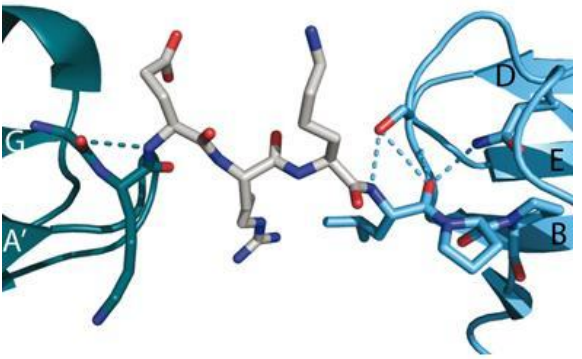

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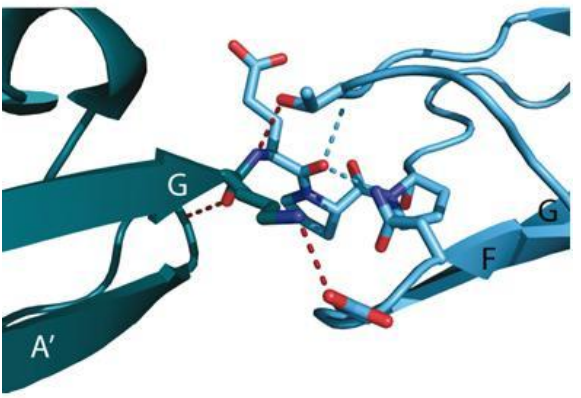

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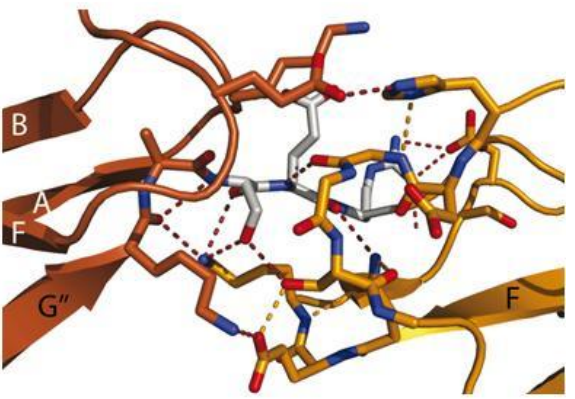

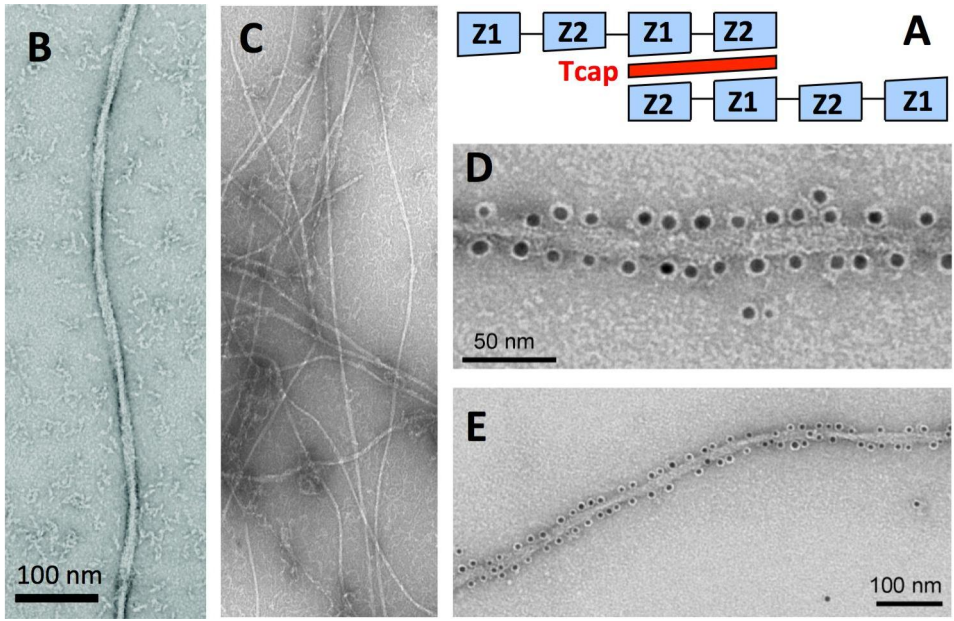
B
100 nm
C
A
Z1
Z2
Z1
Z2
Tcap
Z2
Z1
Z2
Z1
D
50 nm
E
100 nm